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Expression of claudin-7 and loss of claudin-18 correlate with poor prognosis in gastric cancer

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ABSTRACT

Background: The purpose of this study was to evaluate the expression of claudin-3, claudin-7, and claudin-18 in gastric cancer and to determine the significance of these proteins for patient outcome.
Materials and methods: A total of 134 samples were obtained from surgically resected specimens from patients who were diagnosed with gastric carcinoma at a single institution. Paraffin tissue sections from tissue microarray blocks were examined with immunohistochemistry for the expression of claudin-3, claudin-7, and claudin-18.
Results: In normal gastric tissues, positive immunoreactivity was detected for claudin-18 but not for claudin-3 or claudin-7. Claudin-3 and claudin-7 were expressed in 25.4% and 29.9% of the gastric cancer tissues, respectively. However, 51.5% of gastric cancer tissues exhibited reduced expression of claudin-18. Claudin-7 expression was significantly lower in cases with diffuse histologic type and positive lymphatic invasion. There was a significant inverse correlation between claudin-18 expression and perineural invasion. In the survival analysis, the overall survival time was shorter in patients with claudin-7 expression than in those without claudin-7 expression. However, the overall survival was longer in patients with claudin-18 expression than in those without claudin-18 expression.
Conclusions: Our data suggest that the up-regulation of claudin-3 and claudin-7 and the down-regulation of claudin-18 may play a role in the carcinogenesis of gastric cancer. Furthermore, the expression of claudin-7 and the loss of claudin-18 may be independent indicators of a poor prognosis in patients with gastric cancer.

1. Introduction

Gastric cancer is the fourth most frequent malignancy and the second most frequent cause of cancer death in East Asia and the world.[1] Although overall survival has improved in the last few decades, the prognosis of patients with advanced gastric cancer remains poor because tumor progression and metastasis of gastric cancers occur frequently. Advancements have been made in the molecular and histological analysis of most of the cancers arising from the gastrointestinal tract including esophageal, gastric, and colon cancer.[2,3] Despite these remarkable achievements, little diagnostic or therapeutic improvement for patients with cancer recurrence or metastasis has resulted. Therefore, there is a dire need for the identification and characterization of novel molecular markers that can be exploited for determining prognosis.

Claudins, a crucial component of tight junctions, are transmembrane proteins with extracellular loops that are potential targets for diagnostic and therapeutic modalities.[4–6]The alteration in claudin expression might lead to impaired functioning tight junction, have an influence on signaling pathways, and act as a tumor promotional event in some epithelial cancer.[7–9] Recent gene expression profiling analyses have indicated that claudin gene expression is altered in various cancers and claudin protein expression may have significant clinical relevance.[10] Several members of claudin family including claudin-3 and claudin-7 have been reported to be more highly expressed in gastric cancer

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Table 1
Baseline clinical characteristics.

Basic characteristics	Values (%)
Age (year)	63.47 ± 11.64
Gender	
Male	82 (61.2)
Female	52 (38.8)
Histologic type	
Differentiated	71 (53.0)
Less-differentiated	63 (47.0)
Lauren classification	
Intestinal	70 (52.2)
Diffuse	51 (38.1)
Mixed	13 (9.7)
Lymphatic invasion	
Positive	81 (60.4)
Negative	53 (39.6)
Venous invasion	
Positive	30 (22.4)
Negative	104 (77.6)
Perineural invasion	
Positive	55 (41.0)
Negative	79 (59.0)
T stage	
T1	17 (12.7)
T2	24 (17.9)
T3	46 (34.3)
T4	47 (35.1)
N stage	
N0	44 (32.8)
N1	20 (14.9)
N2	29 (21.6)
N3	41 (30.6)
M stage	
M0	122 (91.0)
M1	12 (9.0)
TNM stage	
I	25 (18.7)
II	42 (31.3)
III	55 (41.0)
IV	12 (9.0)
Total cases	134

compared to normal gastric mucosa.[11,12] However, claudin-18 has been reported to be more reduced in gastric cancer compared to normal gastric mucosa.[13] Claudin-low colon cancer is associated with poor survival and this may be also true for gastric cancer.[14]Low claudin-3 and claudin-18 protein expression was associated with poorer survival in an analysis of 94 primary gastric adenocarcinomas.[15] In contrast, in another study high claudin-3 expression in gastric cancer was correlated with longer survival in both univariate and multivariate analyses.[16] Thus, definite correlation between expression and clinical significance of the claudin proteins in gastric cancer remains controversial.

Unfortunately, studies on the prognostic significance of these claudins in gastric cancer have not been extensively studied. In this study, we investigated the expression patterns of claudin-3, claudin-7, and claudin-18 in gastric cancer. In addition, we evaluated the association of the expression of these proteins with the clinicopathological characteristics of gastric cancer and assessed their clinical significance and prognostic value.

2. Patients and methods

2.1. Patients

A total of 134 samples of primary gastric adenocarcinoma were acquired from St. Vincent's Hospital, The Catholic University of Korea from March 2004 to May 2012. An additional 34samples of non-cancerous gastric mucosa were included. The study protocol was approved by the Institutional Review Board of St. Vincent's Hospital, The Catholic University of Korea. The tumors were divided into two histological subgroups: a differentiated type consisting of papillary and well to moderately differentiated tubular adenocarcinomas, and a less-differentiated type consisting of poorly differentiated adenocarcinomas, signet ring cell carcinomas, and mucinous adenocarcinomas. The stages of all of the patients were evaluated in accordance with the guidelines of the Japanese Classification of gastric carcinoma.[17]The surgical treatment comprised gastric resection, according to the localization of the primary tumor, and lymph node dissection following the recommendations of the Japanese Research Society for Gastric Cancer. After surgery, clinical follow-up data were obtained from all of the patients. Survival time was measured as the time from the date of the initial surgery to the date of death.

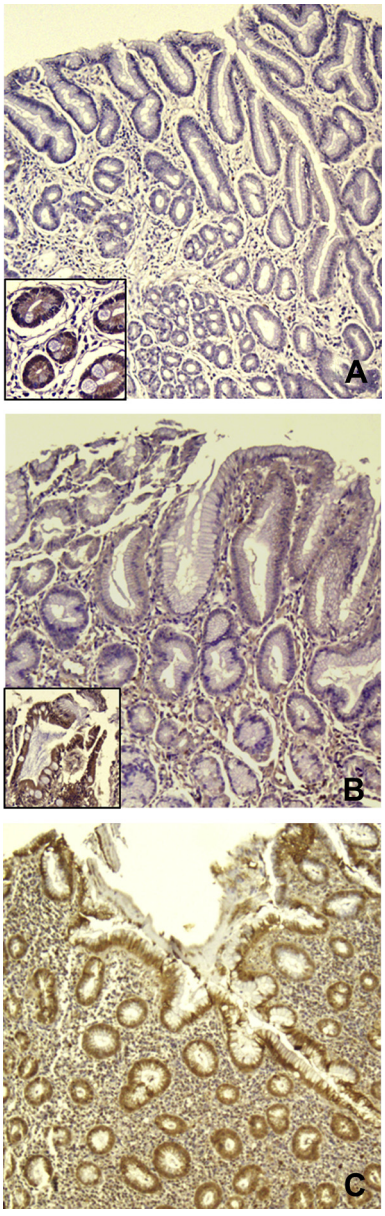

Fig. 1. Immunohistochemicalanalysis of claudin-3, claudin-7, and claudin-18 in normal gastric mucosa. Claudin-3 (A) and claudin-7 (B) are not detected in normal gastric mucosa, however, intestinal metaplastic glands are positive for claudin-3 and claudin-7 (inset)(×100). (C) Expression of claudin-18 is preserved in gastric mucosa (×100).

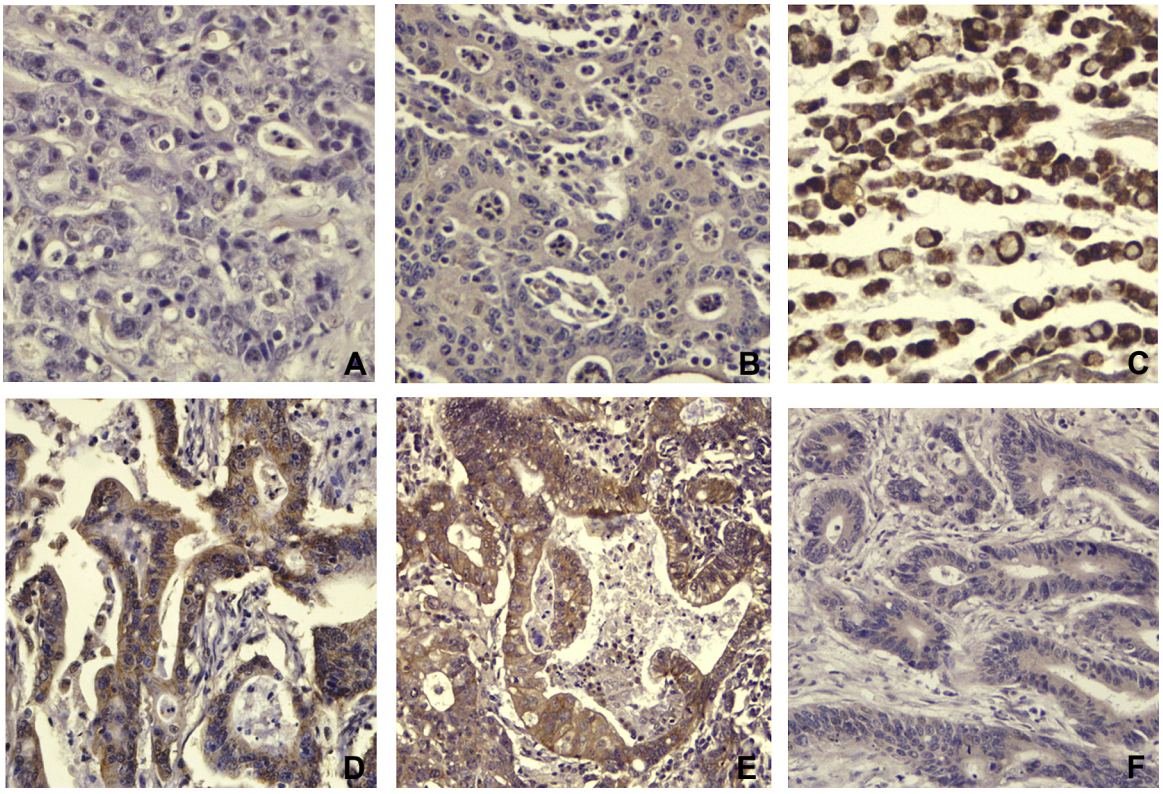

Fig. 2. Immunohistochemicalanalysis of claudin-3, claudin-7, and claudin-18 in gastric adenocarcinoma. (A) Claudin-3 shows negative expression in cancer cells (×200). (B) Claudin-7 shows negative expression in cancer cells (×200). (C) Claudin-18 expression is preserved in gastric cancer cells (×200). (D) Claudin-3 immunostainings show a strong membranous pattern in cancer cells (×200). (E)Claudin-7 immunostainings show a strong membranous pattern in cancer cells (×200). (F) Reduced expression of claudin-18 shows in cancer cells (×200).

Patients who died as a result of the surgery or from other causes were excluded from the study.

2.2. Construction of the tissue microarray (TMA) block

Formalin-fixed paraffin-embedded tissues were obtained from the subjects. Using hematoxylin and eosin (H&E)-stained slides, a representative tumor site was chosen and the site corresponding to the confirmed tumor site in the paraffin block was marked. Areas with necrosis, hemorrhage, or artifacts were excluded. Single core biopsy specimens of 2 mm in diameter were taken from the representative regions (SeongKohn Trader's Co, Seoul, Korea), placed on a TMA mold with 60 pores, and re-embedded with paraffin. The TMA blocks were prepared as 4-μm-thick sections and were stained with H&E. The tissues were then examined to determine whether the appropriate tumor site had been selected.

2.3. Immunohistochemistry

Immunohistochemical staining was performed on 4 μm sections of the tissue microarray blocks using a manual procedure. The paraffin sections were mounted on super frost glass slides, deparaffinized, and rehydrated with a graded series of ethanol, followed by microwave antigen retrieval. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide. The sections were incubated for 1 h or overnight at 4 °C with a 1:100 dilution of a primary rabbit polyclonal antibody against claudin-3 (Abcam, Cambridge, MA, USA), claudin-7 (Abcam, Cambridge, MA, USA), and claudin-18 (Novus, Littleton, CO, USA). Immunostaining was conducted with the rabbit or mouse DAKO ChemMate™ EnVision™ system, Peroxidase/DAB kit (DAKO, Glostrup, Denmark). The sections were then counterstained with Mayer'shematoxylin, dehydrated, cleared, and mounted. Normal intestinal mucosa was used as a positive control for the anti-claudin-3 antibody and colon cancer was used as a positive control forclaudin-7 and claudin-18.

All of the immunostained slides were evaluated independently by two independent pathologists (J.J and H.C). The evaluation was performed twice with the evaluator blinded as to the specific diagnosis and prognosis of each individual case. Staining of cellular membrane with the three antibodies was considered in the evaluation. Claudin-3 and claudin-7 staining were graded according to the number of stained cells and the staining intensity of the individual cells: negative, almost no positive cells or $<$10% of the tumor cells had weak immunoreactivity; or positive, $\geq$10% of the tumor cells had intense immunoreactivity. Claudin-18 staining was considered to be 'preserved' if at least 50% of the cancer cells were stained. When less than 50% of the cancer cells were stained, the immunostaining was considered 'reduced'. The immunohistochemical staining was re-evaluated in cases where there was disagreement between the pathologists. The two pathologists reviewed those cases together, and reached an agreement for the samples with inconclusive results.

2.4. Statistical analysis

The results from the analysis of the continuous variables were expressed as means ± standard deviation (SD). The two-sided *P* values were determined via Chi-square tests. The overall survival of the patients was analyzed using Kaplan–Meier methods with the use of the log-rank test for univariate analysis. The Cox proportional hazards model was used in the multivariate analysis of the factors

Table 2
Correlation between expression of claudin-3, -7, -18 and clinicopathological parameters.

Variables	Cases no	Claudin-3 expression		Claudin-7 expression		Claudin-18 expression	
		Negative	Positive	Negative	Positive	Preserved	Reduced
Age							
<60	46	34(73.9)	12(26.1)	37(80.4)	9(19.6)	19(41.3)	27(58.7)
≥60	88	66(75.0)	22(25.0)	57(64.8)	31(35.2)	46(52.3)	42(47.7)
p Value		0.891		0.060		0.228	
Gender							
Male	82	58(70.7)	24(29.3)	61(74.4)	21(25.6)	43(52.4)	39(47.6)
Female	52	42(80.8)	10(19.2)	33(63.5)	19(36.5)	22(42.3)	30(57.7)
p Value		0.193		0.178		0.253	
Histologic type							
Differentiated	71	51(71.8)	20(28.2)	45(63.4)	26(36.6)	37(52.1)	34(47.9)
Less-differentiated	63	49(77.8)	14(22.2)	49(77.8)	14(22.2)	28(44.4)	35(55.6)
p Value		0.430		0.069		0.375	
Lauren classification							
Intestinal	70	51(72.9)	19(27.1)	42(60.0)	28(40.0)	38(54.3)	32(45.7)
Diffuse	51	41(80.4)	10(19.6)	41(80.4)	10(19.6)	22(43.1)	29(56.9)
Mixed	13	8(61.5)	5(38.5)	11(84.6)	2(15.4)	5(38.5)	8(61.5)
p Value		0.335		0.026		0.359	
Lymphatic invasion							
Absent	53	37(69.8)	16(30.2)	31(58.5)	22(41.5)	24(45.3)	29(54.7)
Present	81	63(77.8)	18(22.2)	63(77.8)	18(22.2)	41(50.6)	40(49.4)
p Value		0.300		0.017		0.546	
Venous invasion							
Absent	104	75(72.1)	29(27.9)	72(69.2)	32(30.8)	51(49.0)	53(51.0)
Present	30	25(83.3)	5(16.7)	22(73.3)	8(26.7)	14(46.7)	16(53.3)
p Value		0.214		0.665		0.819	
Perineural invasion							
Absent	79	55(69.5)	24(30.4)	53(67.1)	26(32.9)	44(55.7)	35(44.3)
Present	55	45(81.8)	10(18.2)	41(74.5)	14(25.5)	21(38.2)	34(61.8)
p Value		0.110		0.353		0.046	
T stage							
T1	17	14(82.4)	3(17.6)	13(76.5)	4(23.5)	8(47.1)	9(52.9)
T2	24	16(66.7)	8(33.3)	16(66.7)	8(33.3)	10(41.7)	14(58.3)
T3	46	32(69.6)	14(30.4)	33(71.7)	13(28.3)	22(47.8)	24(52.2)
T4	47	38(80.9)	9(19.1)	32(68.1)	15(31.9)	25(53.2)	22(46.8)
p Value		0.404		0.893		0.829	
N stage							
N0	44	33(75.0)	11(25.0)	30(68.2)	14(31.8)	23(52.3)	21(47.7)
N1-3	90	67(74.4)	23(25.6)	64(71.1)	26(28.9)	42(46.7)	48(53.3)
p Value		0.945		0.728		0.542	
M stage							
M0	122	90(73.8)	32(26.2)	84(68.9)	38(31.1)	59(48.4)	63(51.6)
M1	12	10(83.3)	2(16.7)	10(83.3.)	2(16.7)	6(50.0)	6(50.0)
p Value		0.468		0.296		0.914	
TNM stage							
I	25	19(76.0)	6(24.0)	18(72.0)	7(28.0)	12(48.0)	13(52.0)
II	42	29(69.0)	13(31.0)	28(66.7)	14(33.3)	21(50.0)	21(50.0)
III	55	42(76.4)	13(23.6)	38(69.1)	17(30.9)	26(47.3)	29(52.7)
IV	12	10(83.3)	2(16.7)	19(83.3)	2(16.7)	6(50.0)	6(50.0)
p Value		0.733		0.727		0.944	

Values are presented as number (%).
*$p < 0.05$.

that were determined to be significant for overall survival with the univariate analysis. $p < 0.05$ was considered to indicate a statistically significant difference with a 95% confidence interval (95% CI).

3. Results

The clinicopathological data of our study population are presented in Table 1. Of the patients, 82 patients (61.2%) were men and 52 (38.8%) patients were women, with a mean age of 63.47 ± 11.64 year (range, 27–85). Twenty-five (18.7%) patients had stage I, 42 (31.3%) patients had stage II, 55 (41.0%) patients had stage III, and 12 (9.0%) patients had stage IV cancer.

The microscopic features of the immunohistochemical staining for each protein are presented in Figs. 1 and 2. In normal gastric mucosa, positive immunoreactivity was detected for claudin-18 but not for claudin-3 and claudin-7. Some intestinal metaplastic epithelia were positive for claudin-3 and claudin-7. Claudin-3 and claudin-7 were expressed in 25.4% and 29.9% of the gastric cancer tissues, respectively. However, 51.5% of the gastric cancer tissues had reduced claudin-18 expression.

The results from the analysis of the correlation between the expression of claudin-3, claudin-7, and claudin-18 and the clinicopathological variables are presented in Table 2. The expression of claudin-7 was significantly higher in cases with intestinal type adenocarcinoma based on the Lauren classification ($p = 0.026$) and in cases without lymphatic invasion ($p = 0.017$). Reduced claudin-18 expression was significantly correlated with perineural invasion ($p = 0.046$).

The survival analyses using the Kaplan–Meier method with regard to the clinicopathological variables and the expression profiles of claudin-3, claudin-7, and claudin-18are summarized in Table 3. Lymphatic invasion ($p = 0.006$), vein invasion

Table 3
Univariate and multivariate analysis of clinicopathologic factors affecting survival rate.

Variables	5-Year survival rate (%)	Univariate analysis *p* value	Multivariate analysis	
			Relative risk (confidence interval)	*p* Value
Age				
<60	61.0	0.105	0.582	0.291
≥60	84.7		(0.213–1.590)	
Gender				
Male	74.9	0.352	0.524	0.178
Female	80.1		(0.204–1.343)	
Histologic type				
Differentiated	80.2	0.532	0.791	0.624
Less-differentiated	73.9		(0.310–2.018)	
Lauren classification				
Intestinal	79.1	0.726	1.379	0.481
Diffuse	72.5		(0.564–3.375)	
Mixed	82.1			
Lymphatic invasion				
Absent	92.9	0.006	1.793	0.417
Present	63.4		(0.438–7.335)	
Venous invasion				
Absent	85.0	0.000	4.614	0.001
Present	44.4		(1.872–11.372)	
Perineural invasion				
Absent	90.3	0.000	5.051	0.003
Present	59.7		(1.753–11.372)	
T stage				
T1	100			
T2	67.4	0.004	1.276	0.498
T3	87.3		(0.630–2.586)	
T4	63.7			
N stage				
N0	93.3	0.014	2.172	0.312
N1-3	69.0		(0.482–9.781)	
M stage				
M0	80.2	0.022	1.463	0.759
M1	34.9		(0.128–16.713)	
TNM stage				
I	100			
II	84.8	0.008	0.649	0.530
III	67.0		(0.168–2.508)	
IV	34.9			
Claudin-3 expression				
Negative	73.4	0.283	0.647	0.523
Positive	86.8		(0.170–2.460)	
Claudin-7 expression				
Negative	79.0	0.101	4.249	0.005
Positive	63.4		(1.544–11.693)	
Claudin-18 expression				
Preserved	90.5	0.082	3.186	0.029
Reduced	64.8		(1.127–9.008)	

*$p < 0.05$.

($p < 0.001$), perineural invasion ($p < 0.001$), the depth of invasion ($p = 0.004$), lymph node metastasis ($p = 0.014$), and the TNM stage ($p = 0.008$) demonstrated a significant correlation with overall survival (OS). With regard to the 3 proteins analyzed, the patients with claudin-7 expression had a shorter OS compared to those without claudin-7 expression (5-year survival rate, 63.4% versus 79.0%)(Fig. 3A). In contrast, the patients with reduced expression of claudin-18 showed significantly worse prognosis than those with preserved expression of claudin-18 (5-year survival rate, 64.8% versus 90.5%)(Fig. 3B). However, overall survival according to claudin-3 was not significantly different (Fig. 3C). Multivariate analysis using the Cox proportional hazards model was performed to evaluate the independent prognostic predictors. As shown in Table 3, the independent prognostic factors that were significantly associated with OS in patients with gastric cancer were venous invasion ($p = 0.001$) and perineural invasion ($p = 0.003$). In addition, the expression of claudin-7 ($p = 0.005$) and loss of claudin-18 ($p = 0.029$) were found to be statistically significant prognostic factors.

4. Discussion

Claudins comprise a multigene family of 27 species and constitute the backbone of the tight junctions that are implicated in

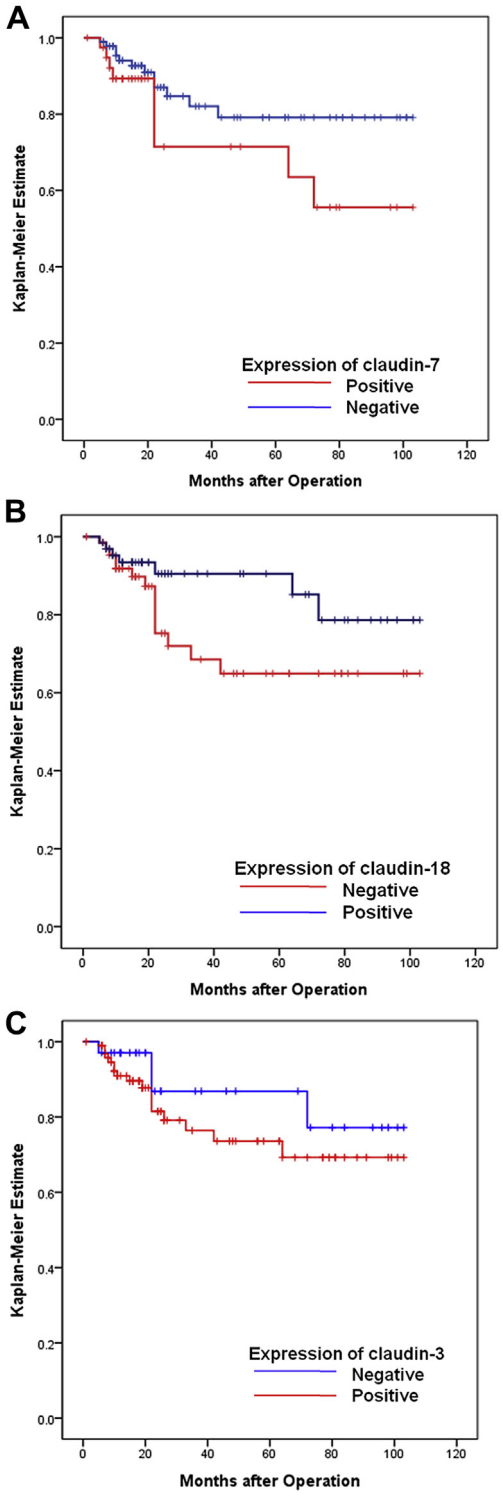

Fig. 3. Survival curves of gastric cancer patients according to expression status of claudin-7 (A) and claudin-18(B). There are significant differences with regard to the expression patterns of claudin-7 and claudin-18.

the adhesive function of epithelial cells.[6] The complex pattern of differentially expressed claudin family members in cancer cells has been reported in previous studies.[16,18] The overexpression or the loss of expression of specific claudin species plays an important role in various malignant diseases.[19–21]

In this study, we determined the expression of claudin-3, claudin-7, and claudin-18 in 134gastric cancer tissue samples with the goal of achieving a more precise understanding of the associations of the expression of these proteins expressions with the clinicopathological characteristics and survival. In this study, we found that claudin-3 and claudin-7 expression was up-regulated not only in cancer cells, but also in intestinal metaplasia, while claudin-18 expression was down-regulated in gastric cancer. These findings are consistent with previous reports.[12,22–25] Notably, we observed a significantly higher frequency of claudin-7 expression in cases with intestinal type adenocarcinoma than in those with diffuse type or mixed type, which is consistent with the results obtained by Johnson et al.[16] and Park et al.[26] and in contrast with those of Erika et al.[25] who found that claudin-7 was expressed mainly in the diffuse type. Regarding the histogenesis of gastric cancer, it has generally been concluded that the differentiated type (intestinal type) carcinomas arise from areas of intestinal metaplasia, whereas undifferentiated (diffuse type) lesions originate from normal gastric mucosa.[3] Therefore, the results that intestinal metaplastic cells and intestinal-type gastric cancer cells expressed highly claudin-7 may support the theory of a carcinogenesis process that progresses from intestinal metaplasia to adenoma to intestinal-type gastric cancer.

Cluadin-18 was first identified as a downstream target gene of the T/EBP/NKX2.1 homeodomain transcription factor.[13] A recent study indicates that claudin-18 is highly expressed in normal gastric cells and down-regulation of this expression is observed in 57.5% of gastric cancers.[24] In our study, we found that normal gastric tissues expressed claudin-18, however, 51.5% of gastric cancers showed reduced claudin-18 expression. Moreover, claudin-18 expression was lower in cases with perineural invasion than in those without perineural invasion, which is a marker for a poor prognosis. These findings suggest that loss of claudin-18 may be related to the aggressive behavior of gastric cancer as well as gastric carcinogenesis. However, further studies are warranted to examine the usefulness of claudin-18 as a prognostic indicator.

There have been few studies reporting the association of survival outcomes with the expression of claudin family members in gastric cancer. Soini et al.[16] reported that claudin-3 expression was associated with a better prognosis of the patients, especially those with the intestinal type cancer. Matsuda et al.[12] demonstrated that the classification of gastric cancers using gastric and intestinal claudins is a good biomarker for assessing the risk of poor prognosis. In a previous study, we demonstrated that tumors expressing claudin-4 were associated with a good prognosis, although we were not able to report 5-year survival results due to the short follow-up periods.[27] In the present study, claudin-7 and claudin-18 had independent prognostic values. Claudin-7 expression was significantly associated with a poorer prognosis of the patients, while the preserved expression of claudin-18was significantly associated with a better prognosis. Our results suggested that the expression profiles of claudin-7 and claudin-18 may be useful prognostic markers in gastric cancer, although this proposal should be studied further to obtain definitive evidence.

Our study has some limitations. First, the clinicopathological characteristics among the cohorts were dissimilar in some parameters. The main reason for these discrepancies is that we conducted a retrospective cohort study to evaluate prognostic significance of claudin-3, claudin-7, and claudin-18. Second, this study has a limitation stemming from its rather small sample size. Third, only the immunohistochemical method was adopted to determine the expressions of claudin-3, claudin-7, and claudin-18. Our results could provide further rationale for its continued investigation in future *in vitro* study.

In conclusion, the increased expression of claudin-3 and claudin-7 and the reduced expression of claudin-18 may play a role in the carcinogenesis of gastric cancer. Moreover, the expression of claudin-7 and the loss of claudin-18 in gastric cancer may be independent markers of a poor prognosis. These findings warrant additional molecular and clinicopathological studies of these markers and their related pathways which are potentially relevant to the prognosis of gastric cancer.

Abbreviations

TMA, tissue microarray; H&E, hematolxyin and eosin; SD, standard deviation; CI, confidence interval; OS, overall survival.

Ethical approval

The study protocol was approved by the Institutional Review Board of St. Vincent's Hospital, The Catholic University of Korea. (VC10TISI0083).

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Author contribution

JH Jung and HM Chin contributed equally to this work; KH Jun, JH Jung, and HM Chin designed the research; JH Kim, JH Jung, and HJ Choi performed the research; HM Chin and JH Jung analyzed the data; KH Jun and JH Jung wrote the paper.

Conflict of interest

None declared.

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